Biological activity of the enantiomers of 3-methylhentriacontane, a queen pheromone of the ant *Lasius niger*

Marine Motais de Narbonne[1], Jelle S. van Zweden[2], Jan E. Bello[3,4], Tom Wenseleers[2], Jocelyn G. Millar[3] and Patrizia d'Ettorre[1*]

[1] Laboratory of Experimental and Comparative Ethology, University Paris 13, Sorbonne Paris Cité, 93430 Villetaneuse, France

[2] Laboratory of Socioecology and Social Evolution, Department of Biology, KU Leuven, Naamsestraat 59 – Box 2466, 3000 Leuven, Belgium

[3] Departments of Entomology and Chemistry, University of California, 900 University Avenue, Riverside, CA 92521, USA

[4] Current Address: Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, Hans-Knoell-Str. 8, 07745 Jena, Germany

* Corresponding author: dettorre@leec.univ-paris13.fr

Key words: social insects, cuticular hydrocarbons, chirality, worker reproduction

Summary Statement:

Queen pheromones regulate division of labor in social insect colonies. Here, we tested the biological activity of the two enantiomers of the black garden ant queen pheromone.

Journal of Experimental Biology • Advance article

ABSTRACT

Queen pheromones are essential for regulation of the reproductive division of labor in eusocial insect species. Although only the queen is able to lay fertilized eggs and produce females, in some cases workers may develop their ovaries and lay male-destined eggs, thus reducing the overall colony efficiency. As long as the queen is healthy, it is usually in the workers' collective interest to work for the colony and remain sterile. Queens signal their fertility via pheromones, which may have a primer effect, affecting the physiology of workers, or a releaser effect, influencing worker behavior. The queen pheromone of the ant *Lasius niger* was among the first queen pheromones of social insects to be identified. Its major component is 3-methylhentriacontane (3-MeC_{31}), which is present in relatively large amounts on the queen's cuticle and on her eggs. 3-MeC_{31} regulates worker reproduction by inhibiting ovarian development. Most monomethyl-branched hydrocarbons can exist in two stereoisomeric forms. The correct stereochemistry is fundamental to the activity of most bioactive molecules, but this has rarely been investigated for methyl-branched hydrocarbons. Here, we tested the bioactivity of the (*S*)- and (*R*)-enantiomers of 3-MeC_{31}, and found that whereas both enantiomers were effective in suppressing worker ovarian development, (*S*)-3-MeC_{31} appeared to be more effective at suppressing aggressive behavior by workers. This suggests that the natural pheromone may be a mixture of the two enantiomers. The enantiomeric ratio produced by queens remains unknown because of the small amounts of the compound available from each queen.

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INTRODUCTION

Reproductive division of labor is one of the defining principles of eusociality (Oster and Wilson, 1978). Consequently, in most eusocial groups only one or a few individuals reproduce, even though all individuals usually possess functional reproductive organs and in principle are still able to successfully reproduce. In hymenopteran species, workers may develop their ovaries and lay unfertilized eggs that develop into haploid males, sometimes even in presence of the queen (Alaux et al., 2007; Cuvillier-Hot et al., 2003; Wenseleers and Ratnieks, 2006a). Males produced from worker-laid eggs have the chance to fertilize virgin queens, which will then found new colonies. However, worker reproduction in the presence of a fertile queen would reduce the overall colony efficiency because reproductive workers do not work, thus harming the collective worker interests as well as those of the queen (Cole, 1984; Wenseleers et al., 2004). In most species, queens or workers eliminate eggs laid by reproducing workers, or attack them directly in a behavior known as policing (Ratnieks and Visscher, 1989; Trivers and Hare, 1976; Wenseleers et al., 2005). Given that worker reproduction may be futile when an effective policing system is in place, in many species this leads workers to respond directly to the presence and fertility of the queen by self-restraining their reproduction, i.e., by not developing their ovaries in queenright colonies (Oi et al., 2015; Wenseleers and Ratnieks, 2006a,b).

The primary method used by the queen to signal her presence to workers is by use of chemical signals known as queen pheromones (Le Conte and Hefetz, 2008). Until recently, only the queen pheromone of the honey bee, *Apis mellifera*, had been identified (Keeling et al., 2003; Le Conte and Hefetz, 2008). In addition to reducing worker ovarian development, queen pheromones also inhibit aggression by workers, so that the compounds have both primer and releaser pheromone functions (Vergoz et al., 2007). In ants and wasps, a number of correlative studies suggested that queen fertility is signaled through a subset of the cuticular lipids, which are composed mostly of long-chain hydrocarbons (Cuvillier-Hot et al., 2004; d'Ettorre et al., 2004; Dietemann et al., 2003; Peeters et al., 1999; Sledge et al., 2001). Recently, this was confirmed for several species of ants, a vespine wasp, and a bumblebee, with queen-characteristic hydrocarbons being shown to inhibit ovary development by workers (Holman et al., 2010a, 2013; Van Oystaeyen et al., 2014).

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In the ant *Lasius niger*, previous work has demonstrated that 3-methylhentriacontane (3-MeC_{31}) acts as the queen pheromone (Holman et al., 2010a). Like the queen pheromone of the honey bee, this pheromone is both a primer (inhibiting worker ovary development) and a releaser pheromone (inhibiting worker aggression). This molecule can exist in two stereoisomeric forms, the (*R*)- and the (*S*)-enantiomers. The correct stereochemistry is often crucial for bioactivity of most natural molecules, and in many species, the presence of traces of the "unnatural" enantiomer can result in a significant loss of bioactivity (Mori, 1998, 2007). For *L. niger*, the bioactivity of the two stereoisomers, however, has not been investigated; to date behavioral experiments have employed only the racemic (i.e., 1:1) mixture of the two isomers (Holman et al., 2010a). The two main possibilities are that only one stereoisomer is active, or that the (*R*)- and (*S*)-enantiomers could both be active, and play the same or different roles. Thus, the goal of our study was to test the bioactivity of the (*R*)- and (*S*)-enantiomers of the queen pheromone of *L. niger* as inhibitors of ovary development in workers, and as modulators of aggression by workers. A recent study showed that in 36 monomethyl-branched cuticular hydrocarbons (MBCHs) isolated from species from nine different orders of insects, the stereochemistry of the MBCHs was conserved, with all of the compounds having the (*R*)-configuration regardless of chain length or methyl branch position (Bello et al., 2015). Thus, we hypothesized that the 3-MeC_{31} queen pheromone of *L. niger* has the (*R*)-configuration. Determining the absolute configuration of the queen-produced hydrocarbon would require extraction and purification of this compound from > 1000 mature queens, based on estimates of the amount present in a single queen. This was not logistically feasible, and so we hoped to indirectly address the question of the likely absolute configuration of the naturally produced 3-MeC31 via bioassays.

MATERIAL AND METHODS

SYNTHESIS OF THE ENANTIOMERS OF 3-METHYLHENTRIACONTANE

Optima grade solvents (Fisher Scientific, Pittsburgh, PA, USA) were utilized for all reactions, work-ups, and purifications. Tetrahydrofuran (THF) was distilled from sodium/benzophenone under an argon atmosphere. ^{1}H and ^{13}C NMR spectra were recorded with a Varian INOVA-400 (400 and 100.5 MHz, respectively) spectrometer (Palo Alto, CA, USA), as $CDCl_3$ solutions. ^{1}H NMR chemical shifts are expressed in ppm relative to residual $CHCl_3$ (7.27 ppm) and ^{13}C NMR chemical shifts are reported relative to $CDCl_3$ (77.16 ppm). Solvent extracts of reaction mixtures were dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation under reduced pressure. Crude products were purified by vacuum flash chromatography or column flash chromatography on silica gel (230-400 mesh; Fisher Scientific). Yields refer to isolated yields of chromatographically pure products. Mass spectra were obtained with a Hewlett- Packard (HP) 6890 GC (Avondale, PA) interfaced to an HP 5973 mass selective detector, in EI mode (70 eV) with helium as carrier gas. The GC was equipped with a DB17-MS column (25 m × 0.20 mm i.d., 0.33 µm film). Reactions with air- or water-sensitive reagents were carried out in oven-dried glassware under argon. Specific rotations were obtained on a Rudolph Autopol IV digital polarimeter (Hackettstown, NJ) as CH_2Cl_2, EtOH, or $CHCl_3$ solutions. Five sequential measurements of each chiral compound were acquired and then averaged to obtain the reported specific rotations.

(R)-2-Methylbutan-1-ol [(R)-1]

Vinyl acetate (41.9 mL, 454 mmol) was added to a solution of racemic 2-methylbutan-1-ol (12.35 mL, 114 mmol) in dry dichloromethane (220 mL) and the mixture was stirred 5 min, then *Pseudomonas fluorescens* lipase (980 mg, 300 units/mmol of substrate, Aldrich Chemical Co.) was added in one portion. The resulting mixture was stirred for 30 h, monitoring the enantiomeric excess of (*R*)-2-methylbutan-1-ol via chiral stationary phase GC. The crude product was chromatographed on silica gel (60 g). Elution with hexane/EtOAc (9:1) afforded 1.88 g of pure (*R*)-2-methylbutan-1-ol (*R*)-**1**, which had the following properties: $[\boldsymbol{\alpha}]_D^{25}$= +13.46 (*c* = 2.5, EtOH); $\boldsymbol{\nu}_{max}$ (neat): 3336 (br m), 2956 (s), 2923 (s), 2855 (s), 1465 (m), 1378 (w), 1032 (s), 938 (w), 908 (w), 842 (w) 723 (w); ^{1}H NMR, ∂_H ($CDCl_3$): 0.89 (6H, m), 1.17 (1H, m), 1.18 (1H, m), 1.87 (1H, broad s, OH), 3.45 (1H, dd, *J*= 11.7 Hz, 4.8 Hz),

3.47 (1H, dd, *J*= 11.8 Hz, 5.3 Hz); ^{13}C NMR, ∂_C ($CDCl_3$): 14.0, 16.8, 27.0, 33.8, 68.5; GC-MS [Column: DB-5MS, 5% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 40-280 ºC (+ 5 ºC/min)]: t_R: 4.51 min (96.5%); MS (70 eV, EI); *m/z*: 87 (1, M^+-1), 70 (35), 56 (100), 41 (70). The enantiomeric excess (ee) was determined by GC analysis using a **β**-DEX225 column [30 m × 0.25 mm id × 0.25 µm film, J&W Scientific, Folsom CA; carrier gas, He; temp: 35-220 ºC (held at 35 ºC for 30 min, then + 5 ºC/ min)]: t_{major}: 43.95 min (100 %).

(R)-2-Methylbutan-1-yl triflate [(R)-2]

Pyridine (1.31 mL, 16.3 mmol) and triflic anhydride (3.34 mL, 19.56 mmol) were added sequentially to a cold (-10 ºC) stirred solution of (*R*)-2-methylbutan-1-ol (*R*)-**1** (1.44 g, 16.3 mmol) in dry CH_2Cl_2 (80 mL). The mixture was stirred at -10 ºC for 1 h and then diluted with pentanes (160 mL) and stirred for 30 min. The resulting mixture was filtered through a plug of silica gel (30 g), and the filter cake was washed with hexanes/CH_2Cl_2 (4:1). The filtrate was concentrated in vacuo to give 3.59 g (quantitative) of (*R*)-**2** as a colorless oil, which was used immediately in the next step without further purification or characterization.

(S)-2-Methylbutan-1-yl triflate [(S)-2]

In the same manner as above (*S*)-2-methylbutan-1-ol (*S*)-**1** (1.45 g, 16.5 mmol; Alfa Aesar) gave 3.63 g (quantitative) of (*S*)-2-methylbutan-1-yl triflate (*S*)-**2** as a colorless oil, which was used immediately without further purification or characterization.

(R)-tert-Butyldimethyl((13-methylpentadecyl)oxy)silane [(R)-3]

To a cold (-40 ºC) solution of (*R*)-2-methylbutan-1-yl triflate (*R*)-**2** (3.59 g, 16.3 mmol) in Et_2O (60 mL) was added dropwise Li_2CuCl_4 (0.394 M, 2 mL, 0.75 mmol, 5 mol % catalyst), and the reaction was stirred 10 min. (11-((*tert*-Butyldimethylsilyl)oxy)undecyl)magnesium bromide (2.0 M, 7.5 mL, 15 mmol) was then added to the reaction mixture over 15 min by syringe pump. The mixture was stirred for 2h at -40 ºC until the Grignard was fully consumed, then warmed to room temperature and quenched with saturated aqueous NH_4Cl (40 mL). The layers were separated and the aqueous layer was extracted with hexanes (2 × 75 mL). The combined organic layers were washed with saturated NH_4Cl (2 × 100 mL) and brine (2 × 100 mL), dried over Na_2SO_4, and concentrated in vacuo. The crude product was chromatographed on silica gel (60 g), eluting with hexane/EtOAc (9:1) to yield 4.39 g (82%) of pure (*R*)-**3** as a colorless oil. (*R*)-*tert*-Butyldimethyl((13-methylpentadecyl)oxy)silane, (*R*)-**3,**

showed the following properties: $[\alpha]_D^{25}$= -3.87 ± 0.013 (*c* = 2.70, CH_2Cl_2); ^{1}H NMR, ∂_H ($CDCl_3$): 0.21 (6H, s), 0.85 (3H, d, *J*=7.8 Hz), 0.89 (3H, pseudotriplet, *J*=7.6 Hz), 0.98 (9H, s), 1.25 (20H, br m), 1.48 (2H, m), 1.52 (1H, m), 3.6 (2H, t, *J*=7.6 Hz); ^{13}C NMR, ∂_C ($CDCl_3$): -1.90, 11.5, 22.0, 26.7, 28.2, 29.3, 29.7, 30.2, 30.5, 31.3, 33.0, 36.5, 38.0, 63.0; GC-MS [Column: DB-5MS, 5% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 100-280 ºC (+10 ºC/min)]: t_R: 16.24 min (98.5%); MS of **3** (70 eV, EI); *m/z*: 299 (41, M^+-57), 171 (1), 143 (5), 129 (2), 111 (6), 97 (17), 89 (21), 75 (100), 57 (42), 41(42).

(S)- tert-Butyldimethyl((13-methylpentadecyl)oxy)silane [(S)-3]

In the same manner as above (*S*)-2-methylbutan-1-yl triflate (3.5 g, 15.9 mmol) gave 4.10 g (76 %) of pure (*S*)-**3** as a colorless oil. $[\alpha]_D^{25}$= +3.95 ± 0.03 (*c* = 2.70, CH_2Cl_2).

(R)-13-Methyl-1-bromopentadecane [(R)-4]

Bromine (1.43 g, 27.6 mmol) was added dropwise to a cold (-10 ºC) solution of PPh_3 (7.38 g, 27.6 mmol) in dry CH_2Cl_2 (100 mL) with vigorous stirring. The reaction was slowly warmed to room temperature over 30 min and stirred another 30 min. (*R*)-*tert*-Butyldimethyl((13-methylpentadecyl)oxy)silane (*R*)-**3** (3.71 g, 11.05 mmol) was then slowly added to the reaction mixture and the resulting solution was stirred 1.5 h. The reaction mixture was diluted with hexanes (200 mL) and filtered through a plug of silica gel (15 g), eluting with hexanes. The eluate was concentrated in vacuo to afford 3.03 g (91.5 %) of (*R*)-13-methyl-1-bromopentadecane (*R*)-**4** as a colorless oil with the following properties: $[\alpha]_D^{25}$= -3.35 ± 0.05 (*c* = 1.90, CH_2Cl_2), ^{1}H NMR, ∂_H ($CDCl_3$): 0.90 (3H, t, *J*= 7.6 Hz), 0.98 (3H, d, *J*=6.8 Hz), 1.31 (20H, broad m), 1.54 (2H, m), 1.65 (1H, m), 1.85 (2H, m), 3.46 (2H, pseudotriplet, J=7.4 Hz); ^{13}C NMR, ∂_c ($CDCl_3$): 11.8, 20.5, 27.1, 28.5, 29.8, 30.0, 30.2, 33.5, 32.1, 33.7, 35.5, 37.8; GC-MS [Column: DB-5MS, 5% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 100-280 ^{o}C (+10 ºC/min)]: t_R: 14.14 min (99.5%); MS of **4** (70 eV, EI); *m/z*: 306 (5, [M^++2]), 304 (5, M^+), 275 (3), 221 (1), 207 (1), 179 (1), 163 (1), 149 (1), 135 (1), 113 (3), 97 (8), 85 (12), 71 (28), 57 (100), 41 (58).

(S)-13-Methyl-1-bromopentadecane [(S)-4]

In the same manner as described above 3.0 g (11.1 mmol) of (*S*)-**3** gave 1.48 g (93% yield, 97.9% ee) of (*S*)-13-methyl-1-bromopentadecane (*S*)-**4** as a colorless oil, $[\alpha]_D^{25}$ = +3.41 ± 0.05

(c = 1.90, CH_2Cl_2). Its spectra were identical to those of (*R*)-**4**.

(R)-29-Methyl-15-hentriacontyne [(R)-5]

n-BuLi (2.89 M in hexanes, 1.32 mL, 3.82 mmol) was added dropwise to a cold (-78 ºC) solution of 1-hexadecyne (682 mg, 3.07 mmol) in dry THF (15 mL) and the resulting mixture was stirred at -78 ºC for 10 min, then at -10 ºC for 30 min. After warming to 25 ºC, (*R*)-**4** (800 mg, 2.63 mmol) dissolved in dry THF (3 mL) was added dropwise. A reflux condenser was added to the reaction apparatus and the mixture was heated to a gentle reflux and stirred overnight. The resulting mixture was then cooled to 25 ºC, quenched with saturated aqueous NH_4Cl, and extracted with hexane. The organic layer was washed with water (2 × 50 mL), aqueous $NaHCO_3$ (2 × 50 mL), and brine (50 mL), dried, and concentrated. Unreacted 1-hexadecyne was removed by Kugelrohr distillation of the crude product (oven temp. 60 ºC, 0.1 mm Hg). The residue was chromatographed over silica gel (50 g). Elution with hexanes gave 890 mg (2.15 mmol, 82%) of (*R*)-**5** as a clear oil. $[\alpha]_D^{25}$ = -3.11 (c = 3.47, CH_2Cl_2); ν_{max} (neat): 2954 (m), 2921 (s), 2852 (s), 1464 (m), 1377 (w), 1251 (w), 1056 (w), 843 (w), 721 (w); ^{1}H NMR, ∂_H ($CDCl_3$): 0.86 (6H, m), 0.91 (3H, d, J=6.4 Hz), 1.1-1.4 (48H, br m), 1.55 (1H, m), 2.35 (4H, m); ^{13}C NMR, ∂_C ($CDCl_3$): 14.32, 18.99, 19.74, 20.9, 26.39, 27.27, 28.61, 29.05, 29.38, 29.58, 29.79, 29.91, 32.12, 33.07, 36.26, 79.56, 81.2; GC-MS [Column: DB-5MS, 5% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 100-280 ºC (+10 ºC/min)]: t_R: 33.52 min (97.3 %); MS of **5** (70 eV, EI); *m/z*: 446 (1, M^+), 417 (16), 355 (2), 324 (1), 281 (1), 225 (3), 197 (2), 141 (3), 113 (2), 85 (11), 71 (25), 57 (100), 41 (88); HRMS (EI) calcd for $C_{32}H_{62}$ (M^+): 446.4852. Found: 446.4858.

(S)-29-Methyl-15-hentriacontyne [(S)-5]

In the same manner as described above 549 mg (2.34 mmol) of (*S*)-**4** gave 846 mg (80% yield) of (*S*)-29-methyl-15-hentriacontyne (*S*)-**5** as a colorless oil. $[\alpha]_D^{25}$ = +3.15 ± 0.05 (c = 2.10, CH_2Cl_2). Its spectra were identical to those of (*R*)-**5**. HRMS (EI) calcd for $C_{32}H_{62}$ (M^+): 446.4852. Found: 446.4847.

(R)-3-methylhentriacontane [(R)-6]

A solution of (*R*)-**5** (800 mg, 1.93 mmol) in hexanes (5 mL) was added to a slurry of 5 % rhodium on carbon (80 mg) and anhydrous Na_2CO_3 (700 mg, 5.2 mmol) in hexanes (10 ml) and the resulting mixture was stirred for 10 h under a slight positive pressure of H_2. The

mixture was filtered through a plug of silica gel and concentrated to afford 763 mg of crude crystalline (*R*)-3-methylnonacosane. Recrystallization from hexane/acetone (1:5, 25 mL) gave 737 mg of (91.5% pure) (*R*)-**6** in 50 % overall yield in 5 steps, mp 34 ºC, $[\alpha]_D^{25}$= -3.05± 0.01 (c = 2.50, CH_2Cl_2). ∂_H ($CDCl_3$): 0.84 (3H, d, *J*= 6.3 Hz), 0.85 (3H, t, *J*= 6.7 Hz), 0.87 (3H, t, *J*= 6.5 Hz), 1.16-1.4 (56 H, broad m), 1.53 (1H, m). ^{13}C NMR, ∂_c (ppm): 11.62, 14.32, 19.45, 22.91, 25.67, 27.36, 29.58, 29.72, 29.93, 30.25, 31.81, 32.16, 34.62, 36.88. GCMS [Column: DB-17MS, 17% phenylmethylsiloxane, 30 m × 0.25 mm id; carrier gas, He; temp: 100-280 ºC (+20 ºC/min)]: t_R: 14.12 min (100%); MS of **7** (70 eV, EI); 421 (54, M^+-29), 407 (1), 393 (6), 379 (1), 365 (1), 351 (1), 337 (1), 323 (1), 309 (2), 295 (2), 281 (2), 267 (2), 253 (2), 239 (3), 225 (3), 211 (2), 197 (3), 183 (3), 169 (6), 155 (7), 141 (10), 127 (12), 113 (17), 99 (23), 85 (55), 71 (70), 57 (100), 43 (45). HRMS (EI) calcd for $C_{32}H_{66}$ (M^+): 450.5165. Found: 450.5159.

(S)-3-Methylhentriacontane [(S)-6]

In the same manner as described above 800 mg (1.93 mmol) of (*S*)-**5** gave 746 mg (93% yield) of (*S*)-3-methylhentriacontane (*S*)-**6** in 53% overall yield. $[\alpha]_D^{25}$= +3.01 ± 0.05 (*c* = 2.10, CH_2Cl_2); mp = 36 ºC. Its spectra were identical to those of (*R*)-**6**. HRMS (EI) calcd for $C_{32}H_{66}$ (M^+): 450.5165. Found: 450.5169.

Treatments

Three types of solutions of synthetic 3-methylhentriacontane in pentane (HPLC grade; Sigma-Aldrich) were prepared: (*R*)-3-MeC_{31} at a concentration of 0.01 $mg.ml^{-1}$, (*S*)-3-MeC_{31} at a concentration of 0.01 $mg.ml^{-1}$ and a racemic mixture of the two isomers at a concentration of either 0.005 $mg.ml^{-1}$ each (for colony replicates A, B and C and for aggression experiments; see below) or 0.01 $mg.ml^{-1}$ each (for colony replicates D, E, F, G, H; see below). The latter two solutions were made in order to either keep constant the concentration of both compounds together or the concentration of each single compound (the response to both solutions was not significantly different, see results). For all bioassays, the treatment solutions were 1) treatment P: the pentane only control, 2) treatment M: racemic mixture of 3-MeC_{31}, 3) treatment R: (*R*)-3-MeC_{31} and 4) treatment S: (*S*)-3-MeC_{31}. A glass dummy (Ø 1 mm × 15 mm) was used as a surrogate queen, onto which 10 µl of one of four different treatment solutions were deposited (i.e., 100 ng of compounds in all cases except replicates

D to H which had 200 ng of compounds for the racemic mixture), depending on the treatment. This dose is biologically relevant because queens have 100-200 ng of 3-MeC_{31} on their cuticles (Holman et al., 2010a).

In all bioassays the treatment order was randomized and the behavioral observations and dissections were conducted blind with respect to treatment.

Bioassay 1: Effect of 3-methylhentriacontane on workers' ovarian development

In April 2014, three mature *L. niger* colonies (colonies A, B, and C) located on the campus of the University of Paris 13 were excavated, and approximately 200 workers per colony were transferred to the laboratory and kept at 24°C, under 50% humidity and a 12:12 day-night cycle. For each colony, four groups of 50 workers were placed in separate plastic boxes (8 × 5 × 4 cm), so that each group (sub-colony) could receive a different treatment. A cotton ball with water was provided for moisture and the ants were fed 3 times a week with apple-honey mixture and *Drosophila* fruit flies.

After an acclimatization period of two days, treatment with synthetic hydrocarbons commenced. Each of the plastic boxes received a glass dummy treated with one of the four different treatment solutions (P, M, R, S) each working day. For each deposition of the treatment solution, the glass dummy was removed from the nest box, cleaned with pentane and placed onto a clean glass surface. Then, 10 µl of the respective treatment solution was applied onto the dummy and, after the pentane had evaporated, the dummy was placed back into the nest box. Each sub-colony was treated 19 times over a period of 26 days. The ants then were dissected to assess the development of their ovaries. In April 2015, the experiment was repeated with 5 additional colonies (D, E, F, G, H).

After the treatment period, workers were frozen at -20 °C for at least 10 min. Ovaries were dissected and ovarian development was scored as 0 for undeveloped ovaries without any developing oocytes or 1 for developed ovaries containing oocytes (we had initially scored ovarian development as in Holman et al., 2010a, but was no difference between a model considering the different scores and the binomial model (0/1), so we retained the simple binomial model). Although in general most of the oocytes produced by workers fail, queenless workers do increase the production of viable oocytes compared to queenright workers (Khila and Abouheif, 2008), therefore the presence of oocytes indicates

reproductive attempts. The proportion of workers with developed ovaries was analyzed using GLMM with binomial errors, with colony as random factor to account for within-colony similarities, and treatment and replicate set (2014 or 2015) as fixed factors. A main effect model, i.e. without an interaction term, proved to have the highest explanatory power, as measured by the Aikaike Information Criterion (AIC).

Bioassay 2: Aggression elicited by synthetic hydrocarbons

Experiment a: tests in neutral arenas

In this experiment, workers from 29 additional queenright laboratory colonies kept at 24°C, 50% humidity, and a 12:12 day-night cycle were tested. These ants were descendants of mated queens collected in July 2012 in Paris. Colonies were housed in plastic boxes (15 x 10 x 3 cm) provided with 2 small tubes: one filled with water, blocked with a cotton wick, to maintain a humid environment; the other, containing only wet cotton at the bottom as a refuge for the queen and workers. Ants were fed three times a week with apple-honey mixture and *Drosophila*. For each trial, five workers were taken randomly from their nest and placed into a circular plastic arena (Ø 7 cm). After 10 min of acclimation, the test began. Each colony was tested with the four different treatments, using different groups of five workers for each treatment. We placed a glass dummy treated with one of the solutions (P, M, R, S) in the center of the arena and observed the behavior of the ants for 3 min, recording the frequency of the behaviors antennation, mandible opening, and biting. Out of these, mandible opening and biting were considered aggressive, whereas antennation was scored as non-aggressive. A trial was disregarded if the ants had no contact at all with the dummy.

Experiment b: tests in queenless nests

In this experiment we used 26 colonies founded in the laboratory, composed of approximately 60 individuals housed in plastic boxes (15 x 10 x 3 cm) as described above. From each colony, the queen with her eggs, pupae, and 10 workers were removed from the nest box the day before the test and placed in a different plastic box (8 x 5 x 4 cm), so the remainder of the workers (approximately 50 workers) were queenless. The test consisted of placing a glass dummy treated with one of the solutions (P, M, R, S) at approximately 1 cm from the entrance of the refuge tube. We then observed and recorded the ant behaviors for 3 min as described above in Experiment 2a. All the treatments were tested in a random

order in each colony and we allowed an interval of at least 1 hour between two consecutive tests in the same colony.

Experiment c: tests in the presence of the queen

Here we tested the colony fragments containing the queens with their 10 workers (see above). We put a treated glass dummy (P, M, R, S) at approximately at 1 cm from the queen and observed the behavior of workers for 3 min, as described above.

All behaviors were recorded using with the software Etholog 2.2 (Ottoni, 2000). The frequencies of behaviors of each of these three sub-experiments were arranged into two columns, non-aggressive and aggressive, and were analyzed using a binomial mixed model, with colony as a random factor to account for within-colony similarities, whereas treatment (P, M, R, S) and sub-experiment (a, b, c) were entered as fixed factors. The model with the highest explanatory power (i.e. the lowest AIC) was a main effect model. Statistical analyses were carried out in R (v 3.1.2, R Development Core Team) using the packages lme4 (v 1.1-7) and effects (v 3.0-3).

RESULTS

SYNTHESIS OF THE ENANTIOMERS OF 3-METHYLHENTRIACONTANE

The synthesis of (*R*)-3-methylhentriacontane (Fig. 1) began with the kinetic resolution of (±)-2-methylbutanol with *Pseudomonas fluorescens* lipase and vinyl acetate in dry CH_2Cl_2 (Barth and Effenberger, 1993). This kinetic resolution enantioselectively esterifies (*S*)-(-)-2-methylalkanols to form the corresponding (*S*)-(-)-2-methylalkyl acetates while leaving the (*R*)-(+)-2-methylalkanols unchanged. (*R*)-(+)-2-Methylbutan-1-ol (*R*)-**1** was isolated from the mixture by column chromatography and the enantiomeric purity was measured by gas chromatography on a β-Dex225 chiral stationary phase column (e.e. > 98%). (*R*)-(+)-2-Methylbutan-1-ol was then treated with triflic anhydride and pyridine to form (*R*)-(+)-2-methylbutan-1-yl triflate **2**, which subsequently underwent a Li_2CuCl_4-catalyzed cross-coupling reaction with 11-(*tert*-butyldimethylsilyloxy)undecylmagnesium bromide to form *tert*-butyldimethyl((13-methylpentadecyl)oxy)silane **3** in 78% yield (Scheme 1) (Cahiez et al., 2000; Wang and Zhang, 2008). Protected alcohol **3** was then converted directly to the corresponding bromide **4** by treatment with Ph_3PBr_2 in dichloromethane at -10 ºC, with Ph_3PBr_2 being prepared *in situ* by addition of Br_2 to Ph_3P in dichloromethane at -78ºC (Aizpurua et al., 1986). Dilution of the reaction mixture with hexanes (3x reaction volume) resulted in the precipitation of triphenylphosphine oxide, which was removed by filtration. The silyl alcohol byproduct was removed from the desired alkyl bromide by Kugelrohr distillation. Alkynylation of **4** with 1-hexadecynyllithium in refluxing THF produced 29-methylhentriacont-15-yne **5** (Buck and Chong, 2001), which was subsequently reduced to the desired methylalkane (*R*)-**6** *via* Rh/C catalyzed hydrogenation (53% overall yield) (Zou and Millar, 2011).

(*S*)-3-Methylhentriacontane (*S*)-**6** was obtained in similar fashion by substitution of commercially available (*S*)-(-)-2-methylbutan-1-ol for (*R*)-(+)-2-methylbutan-1-ol in the first step.

BIOASSAY 1: EFFECT OF 3-METHYLHENTRIACONTANE ON WORKERS' OVARIAN DEVELOPMENT

Worker ovarian development was significantly different between treatments (binomial GLMM with colony as random factor, $\chi^2 = 10.95$, $p = 0.012$; Fig. 2) but not significantly

different between our two sets of experiments (2014 and 2015) (χ^2 = 0.89, p = 0.35). The racemic 3-MeC_{31} treatment (treatment M) yielded a significantly smaller proportion of workers with developed ovaries than the solvent control (treatment P; M vs. P, z = -2.95, p = 0.003). The treatments with the pure (*R*)- and *(S)*-enantiomers also resulted in significant decreases in proportions of workers with developed ovaries compared to the control (R vs. P: z = -2.43, p = 0.015; S vs. P: z = -2.42, p = 0.015). The effect of the two enantiomers appeared to be purely additive. This can be seen from the fact that when treatment was coded as two separate factors for the presence or absence of the two enantiomers, and the effect of the two enantiomers was tested in a full factorial model, there was no significant interaction between the effect of both enantiomers (z = 1.24, p = 0.22).

Bioassay 2: Aggression elicited by synthetic hydrocarbons

We consistently found reduced aggression towards treatment S compared to the control treatment P in all of the three experiments: testing queenright ants immediately after removal from the nest (Experiment 2a, Fig. 3), queenless ants kept separated from the queen for 24 hours (Experiment 2b), and workers with the queen present (Experiment 2c) (binomial GLMM with colony as random factor, S *vs*. P, z = -4.13, p < 0.001). However, this was not the case for the treatments R and M, neither of which were different from the control (R *vs.* P, z = -0.95, p = 0.343; M *vs.* P, z = -0.31, p = 0.76). In a full factorial analysis, the interaction term between experiment and treatment was not significant, but we did find significantly elevated aggression in the presence of the queen (Experiment 2c, using Tukey contrasts, 2c *vs.* 2b, z = 5.66, p < 0.001; 2c *vs.* 2a, z = 4.97, p < 0.001; Fig. 3).

DISCUSSION

The queen pheromone of social insects is hypothesized to elicit both longer term primer effects that result in changes in the physiology of the receiver, and more immediate releaser effects that are expressed as a change in behavior upon perception of the pheromone (for *Lasius* ants, see Holman et al. 2010a, 2013). The primer effects are typically manifested as the inhibition of worker reproduction, whereas the releaser effects include inhibition of aggression and/or display of stereotyped submissive behaviors (e.g., Smith et al., 2015). For instance, for the honey bee, one component of the queen pheromone, (2E)-9-oxodecenoic acid (9-ODA), reduces ovarian development in workers while also inducing retinue behavior (Keeling et al., 2002; Le Conte and Hefetz, 2008).

Our experiments confirmed that the *Lasius niger* queen pheromone component 3-MeC_{31} has both primer and releaser effects, but that these two distinct effects might be mediated by different enantiomers. Both the (*R*)- and (*S*)-enantiomers, as well as the racemic mixture of the two, inhibited ovarian development in field-collected workers when applied for extended periods of time, whereas the (*S*)-enantiomer reduced aggression towards objects treated with it in lab-reared workers (i.e. appeasing workers surrounding the queen). The level of aggressive behavior towards the (*R*)-enantiomer was similar to that shown towards the racemic mixture (treatment M) and the pentane control (treatment P).

It was unexpected that the primer and releaser effects appeared to be mediated by different enantiomers, or that the (*S*)-enantiomer would be active at all because, as noted in the introduction, a recent study has suggested that the large majority of methyl-branched hydrocarbons in insect cuticular lipids are likely to have the (*R*)-configuration (Bello et al., 2015). However, our results are consistent with the study by Sharma et al. (2015) who showed that the sensilla present on the antennae of *Camponotus* ants respond to both (*R*)- and (*S*)-enantiomers and that ants are able to discriminate between these enantiomers in behavioral tests.

We found that the mixture of both enantiomers had a similar effect on the inhibition of workers' ovary development as the (*R*)- and (*S*)-enantiomers alone, suggesting that the enantiomers have an equal, additive effect on ovary development. Our results therefore suggest that the queen-produced pheromone in *L. niger* might actually consist of a mixture

of the two enantiomers. One possible evolutionary scenario that might explain these results is a molecule which initially has evolved a pheromonal function (e.g., primer effect), and then its enantiomer acquires another purpose (e.g., releaser effect). This complex process would be an example of co-option/change (Oi et al., 2015; Holman et al., 2010a; Wyatt, 2014). Alternatively, queens might parsimoniously produce only a single enantiomer, the (*S*) isomer fulfilling both functions. In this case, one would expect to see the other enantiomer having lesser or no effect (depending on the specificity of the receptor for the pheromone), or conversely, this enantiomer should elicit only an aggressive behavioral response if it were simply perceived as unnatural or foreign, analogous to the aggressive responses elicited by the cuticular lipids of non-nestmates (van Zweden and d'Ettorre, 2010; Ozaki and Hefetz, 2014). However, there are cases known in which the non-natural stereoisomers actually have a higher bioactivity than the naturally occurring one (Eliyahu et al., 2004), which would match our results if the (*S*) isomer does not occur naturally in *L. niger*.

There also are several instances known of insects using blends of enantiomers as pheromones (e.g., bark beetles, grain beetles, Lepidoptera; reviewed in Mori, 1998, 2007). For the olive fly, *Dacus oleae*, males and females produce a racemic mixture of their spiroacetal pheromone olean, but the sexes respond differentially to the two enantiomers (Haniotakis et al., 1986). Unfortunately, the technology does not yet exist to determine the enantiomeric ratio of the ~100 ng quantities of 3-MeC_{31} which are present on the cuticle of a *L. niger* queen by analytical chemistry methods (Bello et al., 2015). Thus, our interpretation of the results described above remains somehow speculative, until the enantiomer or blend of enantiomers produced by *L. niger* queens can be conclusively determined.

ACKNOWLEDGEMENTS

Many thanks to Chloé Leroy and Paul Devienne, University of Paris 13, for technical assistance. We thank two anonymous reviewers for useful comments on the manuscript. The study was supported by a Marie Curie Reintegration Grant to PdE (FP7-MC-ERG-2009-256524) and a postdoctoral fellowship of the Research Foundation Flanders to J.S.vZ. (FWO 12Q7615N).

COMPETING INTERESTS

The authors declare no competing or financial interests.

AUTHOR CONTRIBUTIONS

M.M.dN., J.S.vZ. and P.dE. conceived and designed the experiments; J.E.B. and J.G.M. synthesized the chemical compounds; M.M.dN. conducted the experiments; J.S.vZ. and T.W. analyzed the data; M.M.dN. and P.dE. drafted the manuscript; all authors revised the manuscript.

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FIGURES

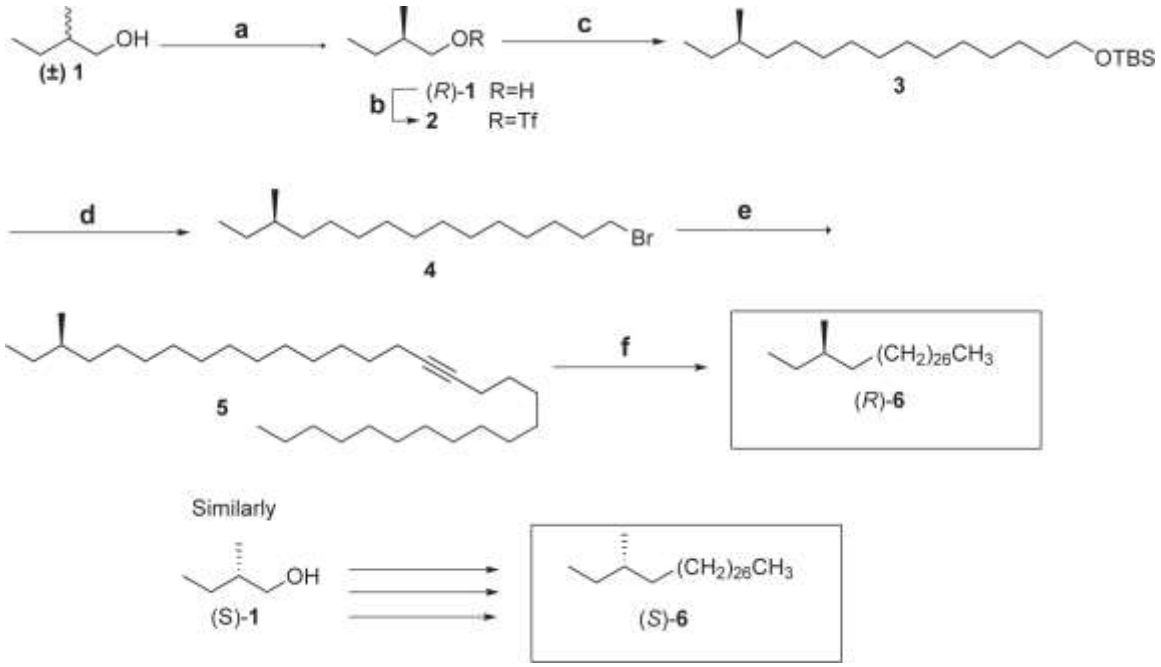

Figure 1. Synthesis of (*R*)-3-methylhentriacontane [(*R*)-**6**], and (*S*)-3-methylhentriacontane [(*S*)-**6**]. **(a)** *Pseudomonas fluorescens* Amano lipase, vinyl acetate, CH_2Cl_2; **(b)** Tf_2O, pyridine, CH_2Cl_2 (quantitative); **(c)** 11-(*tert*-butyldimethylsilyloxy)undecylmagnesium bromide, Li_2CuCl_4, Et_2O (78 %); **(d)** Ph_3PBr_2, CH_2Cl_2 (92 %); (e) hexadecynyllithium, THF, reflux (85 %); **(f)** 5 % Rh/C, H_2, hexane (92 %, 53% overall yield).

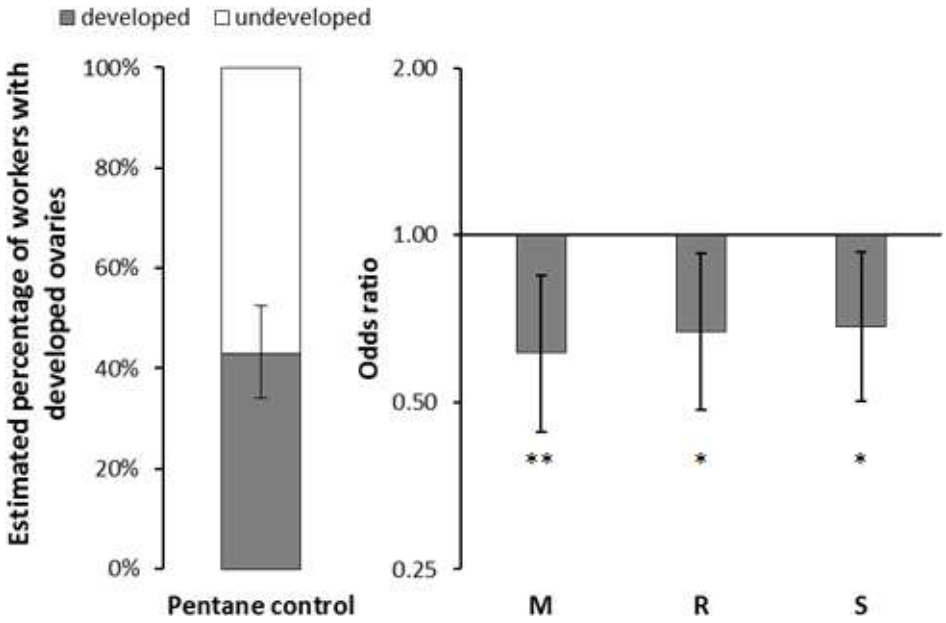

Figure 2. Effect of the two enantiomers and the racemic mixture of the queen pheromone 3-MeC31 on worker ovary development. Effects are presented as the ratio of the odds that workers developed their ovaries (right panel) compared to the pentane control condition (left panel; n = 8 colonies / 351 dissected workers) as estimated from a GLMM. Error bars depict 95% confidence intervals. A significant difference is inferred if the 95 % CI of the odds ratio does not include 1. M: racemic mixture (n = 8 colonies / 306 dissected workers), R: (R)-enantiomer (n = 8 colonies / 309 dissected workers), S: (S)-enantiomer (n = 8 colonies / 369 dissected workers), *: $p < 0.05$, **: $p < 0.01$.

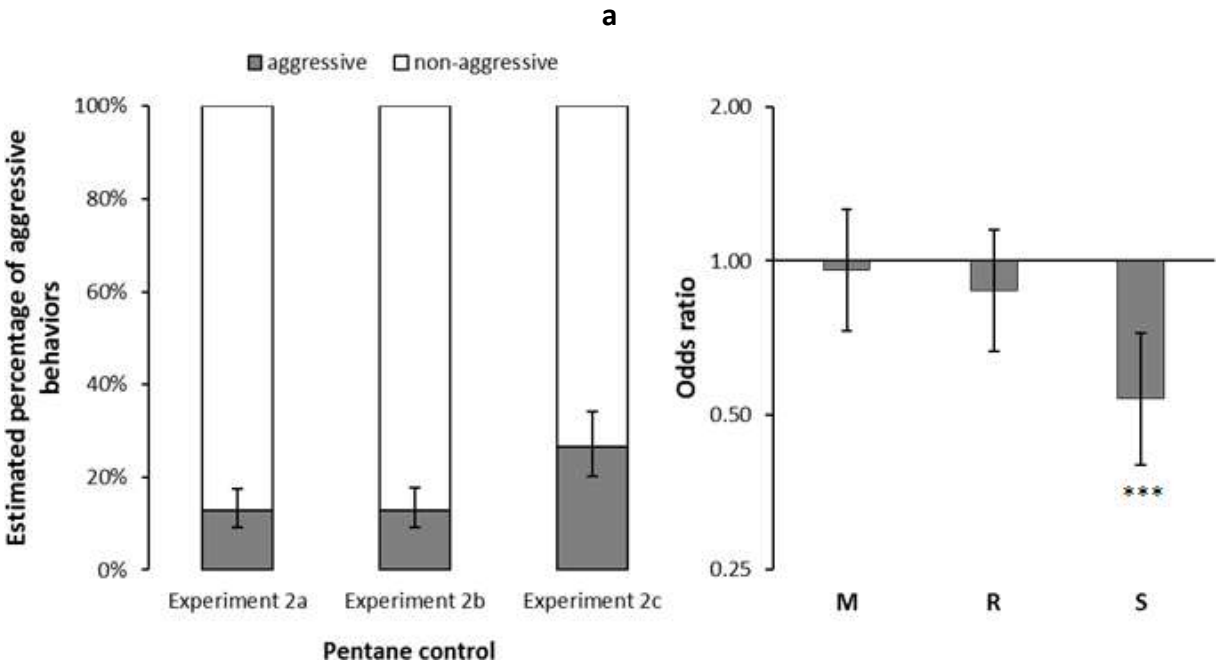

Figure 3. Effect of the two enantiomers and the racemic mixture of the queen pheromone 3-MeC31 on worker aggressive responses. Effects are presented as the ratio of the odds that workers displayed aggressive behavior (right panel) compared to the pentane control condition (left panel; n = 68 replicates / 785 observed behaviors) as estimated from a GLMM. There was significantly elevated aggression in Experiment 2c (Experiment 2a vs. 2c, $z = 7.317$, $p < 0.001$; Experiment 2b vs. 2c, $z = 6.682$, $p < 0.001$). Experiment 2a tested queen-right ants immediately after removal from the nest, 2b tested queenless ants kept separated from the queen for 24 hr, and 2c tested workers with the queen present. Error bars depict 95% confidence intervals. A significant difference is inferred if the 95 % CI of the odds ratio does not include 1. M: racemic mixture (n = 64 replicates / 712 observed behaviors), R: (R)-enantiomer (n = 69 replicates / 744 observed behaviors), S: (S)-enantiomer (n = 68 replicates / 693 observed behaviors), *: $p < 0.05$, **: $p < 0.01$.